Site-directed mutagenesis of squalene–hopene cyclase: altered substrate specificity and product distribution

Tongyun Dang and Glenn D Prestwich

Background: Two regions of squalene–hopene cyclase (SHC) were examined to define roles for motifs posited to be responsible for initiation and termination of the enzyme-catalyzed polyolefinic cyclizations. Specifically, we first examined the triple mutant of the DDTAVV motif, a region deeply buried in the catalytic cavity and thought to be responsible for the initiation of squalene cyclization. Next, four mutants were prepared for Glu45, a residue close to the substrate entrance channel proposed to be involved in the termination of the cyclization of squalene.

Results: The DDTAVV motif in SHC was changed to DCTAEA, the corresponding conserved region of eukaryotic oxidosqualene cyclase (OSC), by the triple mutation of D377C/V380E/V381A; selected single mutants were also examined. The triple mutant showed no detectable cyclization of squalene, but effectively cyclized 2,3-oxidosqualene to give mono- and pentacyclic triterpene products. Of the Glu45 mutants, E45A and E45D showed reduced activity, E45Q showed slightly increased activity, and E45K was inactive. A normal yield of pentacyclic products was produced, but the ratio of hopene **2** to hopanol **3** was significantly changed in the less active mutants.

Conclusions: Initiation and substrate selectivity may be determined by the interaction of the DDTAVV motif with the isopropylidene of squalene (for SHC) and of the DCTAEA motif with the epoxide of oxidosqualene (for OSC). This is the first report of a substrate switch determined by a central catalytic motif in a triterpenoid cyclase. At the termination of cyclization, the product ratio may be largely controlled by Glu45 at the entrance channel to the active site.

Department of Medicinal Chemistry, The University of Utah, 30 South 2000 East, Room 201, Salt Lake City, UT 84112-5820, USA

Correspondence: Glenn D Prestwich
E-mail: gprestwich@deans.pharm.utah.edu

Keywords: Site-directed mutagenesis; Triterpene biosynthesis

Received: **1 March 2000**
Revisions requested: **29 March 2000**
Revisions received: **26 May 2000**
Accepted: **7 June 2000**

Published: **1 August 2000**

Chemistry & Biology 2000, **7**:643–649

1074-5521/00/$ – see front matter

PII: S1074-5521(00)00003-X

Introduction

Squalene–hopene cyclases (SHCs) and oxidosqualene cyclases (OSCs) are the key enzymes in triterpenoid biosynthesis that convert the acyclic precursors squalene (**1**) and (3*S*)-2,3-oxidosqualene (OS, **4a**) to polycyclic products (Figure 1) [1,2]. These intriguing regio- and stereospecific polyolefinic cyclizations [3–5] have been a focus for biological chemists seeking selective new mechanistic insights and medicinal chemists developing selective inhibitors as potential hypocholesteremic drugs [1,2,6].

Cyclization is believed to proceed through a series of discrete conformationally rigid, partially cyclized carbocationic intermediates [1,5]. Overall, the theme of 'template, trigger, chaperone, and sequester' is maintained in both cyclases [7]. The substrate first binds to the enzyme by adopting a specific 'product-like' conformation and then triggers cation formation by protonation of a double bond or an epoxide. Next, the cyclase acts as a chaperone for reactive carbocationic intermediates as they undergo sequential ring closure and rearrangement reactions; during this process, cyclization intermediates are sequestered and stabilized [7,8], a process that involves π–cation interactions [9,10], as discussed by Dougherty [11]. Finally, deprotonation of the C-*22* cation by SHC yields cyclization products (Figure 1A).

Analysis of 2.9 Å and 2.0 Å resolution three-dimensional crystal structures for SHC revealed two α–α-barrel domains that assemble to form a central hydrophobic cavity [12,13]. These α-barrel domains are topologically distinct from the single α-helical bundles that characterize the sesquiterpene cyclases [8]. Domain 1 is an α6–α6 barrel of two concentric rings of parallel helices, while domain *2* appears to have evolved from domain 1 by gene duplication and loss of one of the helices [12]. SHC has a central cavity of 1200 $Å^3$, in which the catalysis is presumed to take place.

Site-directed mutagenesis of Asp376 and Asp377 of the SHC DDTAVV motif showed that both acidic residues are required for catalysis [14]. Asp376 of SHC was proposed to be the electrophilic residue that initiated cyclization by protonating the terminal isopropylidene of squalene, based on the three-dimensional structure of SHC containing an inhibitor, *N*,*N*-dimethyldodecylamine *N*-oxide, in the active site [12]. Although this DDTAVV motif is

highly conserved in all SHCs [15], the role of this motif in initiation has not been verified.

One orally active drug candidate, Ro48-8071, is both photoactivatable and a potent inhibitor of both OSC and SHC [16,17]. This radiolabeled drug photocovalently modified Ala44 in *Alicyclobacillus acidocaldarius* SHC [18]. Molecular modeling based on this result and the published crystal structures [12,13] suggested that Ro48-8071 may bind at the juncture between the central cavity and substrate entrance channel, thus inhibiting access of the substrate to the active site [18]. This region, including Glu45, had been postulated to be important in the termination of cyclization [12,18].

Combining studies of enzyme inhibition, product analyses for mutants and alternative substrates, and the availability of 2.9 Å and 2.0 Å resolution crystal structures of SHC [12,13] now permits targeted approaches to identify residues involved in catalysis. We describe herein product analyses for SHC mutants that implicate specific functions in substrate selectivity and cyclization initiation for the DDTAVV motif, and cyclization termination for Glu45.

Results

The DDTAVV motif in bacterial SHCs is homologous to the conserved DCTAEA motif in plant, yeast, and vertebrate OSCs, and is crucial for cyclase activity [14,19,20]. To test the role of this motif in cyclization, we altered the DDTAVV motif to DCTAEA by preparing a D377C/V380E/V381A triple mutant. This SHC triple mutant was photocovalently labeled by [^{3}H]Ro48-8071 (Figure 2), but cyclization of squalene could not be detected in incubation when assayed at 35°C to 70°C (see Table 1). Absence of squalene cyclase activity is consistent with the observation that the D377E mutant of SHC had only 0.04% wild-type (wt) activity [14].

Interestingly, however, this triple mutant of SHC showed significant cyclization of [^{14}C]2,3-oxidosqualene as detected by radio-thin layer chromatography (radio-TLC). Since wt SHC was known to cyclize both enantiomers of OS to give the diastereomeric hop-22(29)-en-3-ols as products [21,22], a preparative incubation of (3*RS*)-OS with *Escherichia coli* cell lysate for the triple mutant was performed. Gas chromatographic (GC) analysis showed the presence of four cyclization products with retention times (rt) of 12.7, 12.8, 31.0, and 32.3 min. Purification to homogeneity on SiO_2 afforded isolated yields of 10% (for the two materials at 12.7 and 12.8 min), and 10% each for the longer rt products; unreacted substrate was also recovered. The products at 31.0 and 32.2 min were determined to be the diastereomers of hop-22(29)-en-3-ol by high resolution mass spectroscopy (HRMS; calcd. 426.3862, found 426.3864 and 426.3866, respectively), low resolution mass spectroscopy (LRMS; 70 eV, database from mass spectroscopy facility), and nuclear magnetic resonance (NMR; 500 MHz, $CDCl_3$) [23–25]. Specifically, diagnostic chemical shift and coupling patterns for H-3 at either δ 3.12 (dd, $J = 11$, 4.5 Hz, 1H) or 3.32 (m, 1H) supported the 3β-ol (**6b**) and 3α-ol (**6a**) assignments for the longer and shorter rt fractions, respectively. In addition, the angular methyls at δ 0.69 (s, 3H, 4β-Me) and 0.86 (s, 3H, 4α-Me) in **6b** shift to δ 0.76 and 0.87 in **6a**.

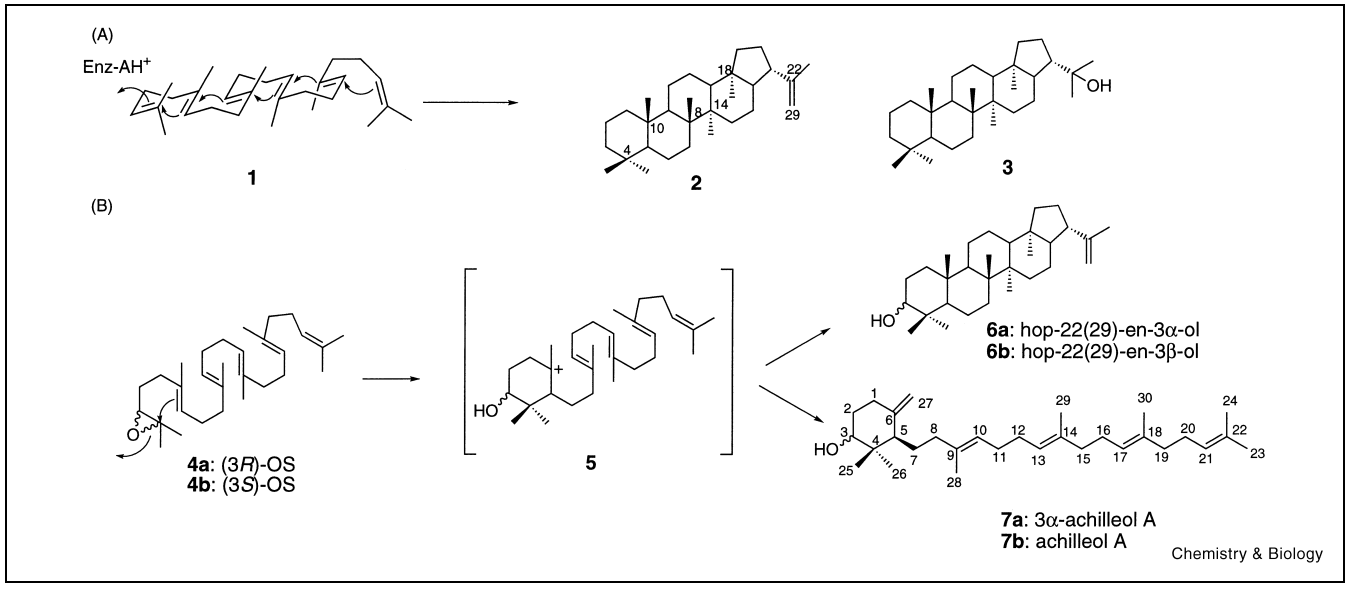

Figure 1. Cyclization by wt and mutant SHC. **(A)** Cyclization of squalene (**1**) to hop-22(29)-ene (**2**) and hopan-22-ol (**3**) by wt SHC. **(B)** Cyclization of (3*RS*)-2,3-OS by SHC mutant D377C/V380E/V381A with conversion to hop-22(29)-en-3α-ol (**6a**) or 3β-ol (**6b**) and 3α-achilleol A (**7a**) or achilleol A (**7b**).

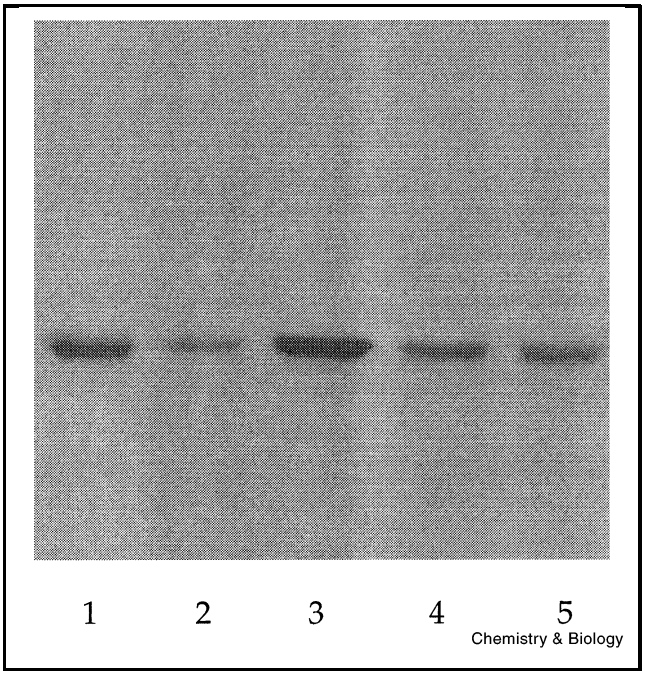

Figure 2. Fluorogram illustrating photoaffinity labeling of SHC mutants with [^{3}H]Ro48-8071. Equal amounts of proteins were loaded into each lane after irradiation [17]. After SDS–PAGE separation, labeled protein was visualized by fluorography. **Lane 1**, E45A; **lane 2**, E45D; **lane 3**, E45K; **lane 4**, E45Q; **lane 5**, D377C/V380E/V381A.

The two compounds at rt 12.7 and 12.8 min could not be resolved chromatographically and were examined as a mixture. HRMS indicated the formula $C_{30}H_{50}O$ (calcd. 426.3862, found 426.3868) for both isomers. The presence of *exo*-methylene groups and trisubstituted alkene vinyl protons indicated that incomplete cyclization had occurred. Comparison of the NMR data of ^{1}H, ^{1}H–^{1}H COSY, HMQC at 500 MHz with literature data [26,27], in conjunction with LRMS fragmentation patterns (70 eV), allows structural assignment as the diastereomeric compounds achilleol A (**7b**, Figure 1B) and 3α-achilleol A (**7a**, Figure 1B). Since wt SHC cyclized squalene into hop-22(29)-ene and gave hopan-22-ol as byproduct, hopan-3,22-diol could conceivably be produced as a minor product from (3*RS*)-OS by either wt or mutant SHC [21]; however, no diol products were detected.

To determine which residues could account for the change of the substrate specificity and the interruption of the cyclization at the monocyclic stage, single mutations of D377C, V380E, and V381E were also constructed, tested, and the cyclization products were analyzed by GC. When squalene was tested as substrate, D377C had negligible activity, but both V380E and V381A had cyclase activity comparable to that of wt SHC. When using OS as substrate, all three single mutants cyclized OS into hop-22(29)-en-3-ols. Interestingly, no achilleol A or 3α-achilleol A was detected as product for V380E and V381A, while traces of monocyclic products were detected for D377C.

Four single-point mutants of SHC (E45A, E45D, E45K and E45Q) were constructed, expressed, purified and assayed. Table 1 shows the enzymatic activity of the wt enzyme and four mutants at 60°C, the optimal temperature for wt SHC. The E45A mutant retained 6% wt activity, while the E45K mutant showed no detectable activity. Shortening the side chain of the Glu residue to Asp in E45D resulted in 42% wt activity. Most intriguingly, replacement of the glutamate with a glutamine in E45Q gave 118% wt activity. On the other hand, K_m values of wt and mutants for squalene were essentially the same as that previously reported for wt SHC [28], indicating that the mutation of E45 did not affect the binding of squalene. Thus, the difference of the activity observed for mutants suggested the catalytic role of Glu45 (Table 1). Each of the mutants was efficiently labeled by [^{3}H]Ro48-8071, regardless of catalytic activity, also suggesting that the SHC mutants are all properly folded (Figure 2).

SHC cyclizes squalene into hop-22(29)-ene (**2**) and hopan-22-ol (**3**) (Figure 1A). Analysis of the products by capillary GC (Table 1) revealed that more hopanol (**3**) is formed relative to hopene (**2**) for E45A and E45D mutants in comparison to wt and E45Q. This supports an earlier suggestion that Glu45 could be involved in stabilization of a C-22 carbocation intermediate [12,13,18]. That is, as in E45A and E45D, the absence of stabilization of the terminal cation by an appropriately positioned nucleophilic group facilitates quenching of the cyclization by water, while the E45Q permits stabilization to achieve a normal production ratio. Importantly, the complete lack of activity of E45K suggests that the reversal of charge near the ter-

Table 1
Properties of SHC mutants. Squalene was used as substrate and all activity was compared to wt SHC.

SHC	Relative activity at 60°C (% wt)	K_m (μM)	Optimal temperature (°C)	Ratio of **2**:**3**
wt	100.0	4.0	60	7.6
E45A	5.9	4.5	50	3.6
E45D	42.2	4.1	50	4.1
E45K	N.A.	n.d.	tr	n.d.
E45Q	118.3	4.6	55	8.3
D377C/V380E/V381A	N.A.	n.d.	N.A.	n.d.

N.A., no detectable activity; n.d., not determined; tr, traces of pentacyclic products at lower temperatures.

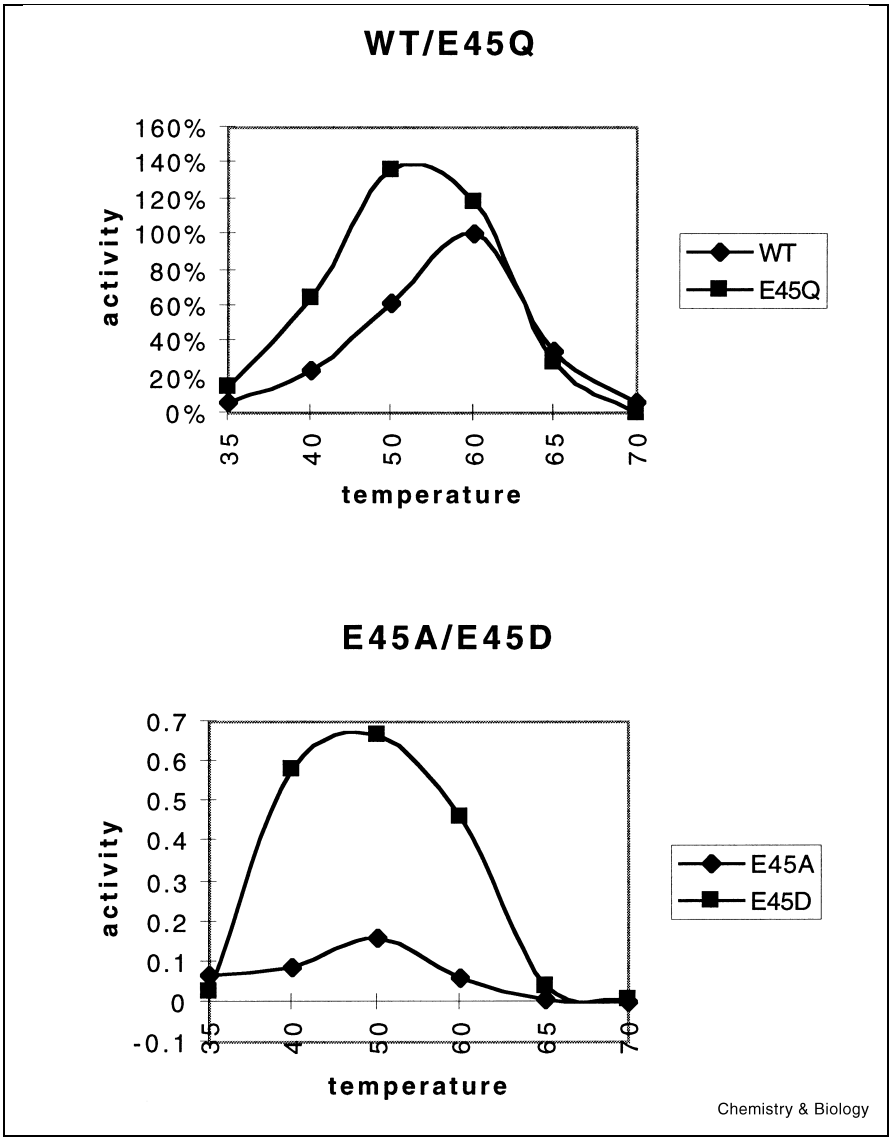

Figure 3. Temperature vs. SHC activity profiles for Glu45 mutants. Squalene was used as the substrate and product analysis was by GC.

mination of cyclization would remove an important source of carbocation stabilization and quenching, and thus prevent cyclization from being initiated.

Temperature profiles for cyclization by mutant and wt SHCs were obtained by measuring squalene cyclization at 35, 40, 50, 60, 65, and 70°C. Incubation times were adjusted to give equivalent net conversion to product. As indicated in Table 1 and Figure 3, both E45A and E45D showed maximal activity at 50°C, which was 10°C lower than for wt SHC.

Discussion

The acidic residues Asp376 and Asp377 are crucial residues for SHC activity [14,19,20], but the identity of the residue likely to initiate cyclization by isopropylidene protonation is still in question. Cyclization of OS, but not squalene, by the triple mutant D377C/V380E/V381A offers strong support for protonation of the epoxide by Asp376. The higher resolution SHC structure shows two H-bonded pairs present in proximity to each other: Asp376:His451 and Asp374:Asp377 (Figure 4) [12]. It appears that the negative charge borne by Asp374:Asp377 stabilizes the positive charge on Asp376:His451 [12] (Figure 2), since Asp alone may not have sufficient acidity to initiate the cyclization [20]. The function of Asp376 in SHC could be further enhanced by a proximal water molecule [13].

In the absence of this reinforcing interaction, as in the triple mutant D377C/V380E/V381A, it seems that the protonated carboxylic acid of Asp376 alone is no longer able to protonate an alkene. Nonetheless, consistent with the

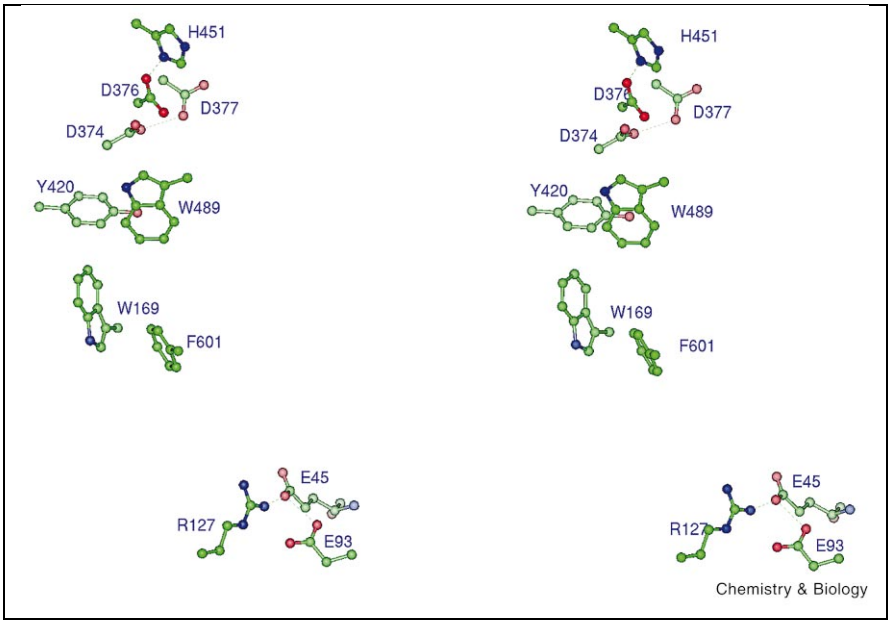

Figure 4. Stereopairs illustrating the residues that contribute to the initiation (Asp374, Asp376, Asp377, His451) and termination (Glu45, Glu93, Arg127) of squalene cyclization by SHC. The positive charge on Asp376:His451 is stabilized by the negative charge from Asp374:Asp377 to initiate the cyclization. The carbocation intermediate formed at the cyclization termination is stabilized by a network constituted by Gln262:Glu45:Glu93:Arg127. For comparison, Trp489, Phe601, Tyr420, and Trp169 are also shown; these residues are reportedly involved in stabilizing cyclization intermediates [9,10,44].

DCTAEA motif as a requirement for OSC function in eukaryotic enzymes, the triple mutant can still initiate cyclization of OS by protonating the 2,3-epoxide. However, due to the possible structural changes introduced by the mutation of these three residues proximal to the initiation site, the carbocationic intermediate appears to partition between complete cyclization and early termination (Figure 1B). This result lends support to the hypothesis that oxirane ring cleavage and A-ring formation occur in a concerted process [29–31]. On the other hand, the enzyme–substrate complex involves many interactions, and even subtle disturbance of the chaperoning effect can alter the cyclization process. For example, mutation of Ile481 in cycloartenol synthase from *Arabidopsis thaliana* (homologous residue to Asp374 in SHC) to Val481 generated lanosterol and parkeol in addition to cycloartenol (the usual exclusive cyclization product) in a ratio of 26:22:52 [32]. Interestingly, mutation of Val454 in lanosterol synthase from *Saccharomyces cerevisiae* (also homologous to Asp374 in SHC) to Ala or Gly also gave achilleol A as a byproduct, in addition to lanosterol [33]. These results with OSCs demonstrated that small steric differences of these aliphatic residues [32,33] could influence either final deprotonation (lanosterol vs. cycloartenol) or continuation of cyclization. In a different approach, the N-terminal domain of a plant triterpene cyclase was found to be product-determining using chimeric OSC constructs [34].

Although A-ring formation is most likely a concerted process, the subsequent steps require stabilization of cationic intermediates by aromatic or nucleophilic residues within the active site. For example, mutation of the residues that stabilize later intermediates, such as Y420A (Figure 4), gave rise to bi- and tricyclic products [9,10]. Similarly, partial cyclization to mono- and bicyclic products by wt SHC was observed using 11-fluoro- and 14-fluoro-substituted OS as substrates [35,36].

Alteration of the π-cation stabilizing residues can also affect the outcome of the cyclization. Thus, mutagenesis of three conserved tyrosine residues (Tyr495, Tyr609, and Tyr612) in *A. acidocaldarius* SHC was reported very recently, and each Tyr residue was shown to be intimately involved in the cyclization process [37]. Y612F had reduced activity compared to wt SHC, while Y495F was essentially inactive. However, both mutants gave the same product pattern as wt SHC. In contrast, Y609F had an activity comparable to wt SHC, but one bicyclic and five tetracyclic triterpenes were also isolated as cyclization products in addition to hopene and hopanol. This result suggested that Tyr609 contributed to the stabilization of carbocation intermediate(s), and that the altered interaction with Phe substitution led to early cyclization termination.

An independent report recently described a functional role for the $D^{374}XDDTAVV^{381}$ motif during SHC cyclization [38], in which mutagenesis data also supported the importance of Asp376 and His451 in cyclization initiation. On the other hand, the authors argued that Asp377 acted to stabilize C-10 carbocation intermediate (hopene numbering). However, this hypothesis appears not be consistent with the three-dimensional active site structure, in particular the hydrogen bonding between Asp374 and Asp377 [12]. It is also inconsistent with the models previously described [13].

While Asp376 and Asp377 are located deep within the hydrophobic catalytic cavity, Glu45 is located near the sub-

strate access or product exit channel (Figure 4) [12]. The reduction of cyclization activity for E45D and E45A, and the abrogation of cyclization activity for E45K, confirm a role for Glu45 in the cyclization process. This residue, which is conserved in all SHC sequences, provides a nucleophilic group important in cyclization termination. This is consistent with earlier findings that a hydrogen-bonding network of Gln262:Glu45:Glu93:Arg127 involving also a water molecule may stabilize or quench the C-22 carbocation [12,13]. Although E45D has a carboxylate functionality, its reduced activity showed that not only nucleophilicity, but also positioning of the carboxylate group of Glu45, was important. The enhanced activity of E45Q relative to wt SHC is not as readily explained. In other enzymes for which glutamic acid residues are purported to be responsible for stabilization of carbocation intermediates, substitution of glutamine generally decreased activity [39,40]. Examination of the interaction between the side chain of Glu45 and proximal residues showed that hydrogen bonding was limited to only one of the carboxylate oxygens (with Arg127 and Glu93, see Figure 4). This H bond is retained in E45Q. We conclude that, although Glu45 is important in stabilizing the terminal carbocation, it may not be the only residue involved in cyclization termination.

Significance

Although triterpene cyclization has been studied for many years [1], detailed studies of the enzyme–substrate interaction were not possible until three-dimensional structures of SHC became available [12,13]. Targeted mutations in putative catalytic motifs conserved with different sequences in eukaryotic OSCs and prokaryotic SHCs reveal that substrate selectivity for cyclization initiation resides in the DCTAEA (OSC) or DDTAVV (SHC) motifs. Moreover, a glutamic acid at the distal end of the cyclase active site appears to provide subtle guidance of a water molecule in the product determination step. Due to the similarity between OSC- and SHC-catalyzed cyclization, and absence of a three-dimensional structure for OSC, rich information can still be obtained from SHC. Such insight enhances design of additional cholesterol-lowering and anti-fungal drugs.

Materials and methods

Plasmid construction

A pUC19-derived plasmid encoding His_6-tagged SHC from *A. acidocaldarius* was used as template for the site-directed mutagenesis [14]. Mutated DNA was constructed by PCR amplification using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). The triple mutant D377C/V380E/V381A was constructed by replacing GAC by TGC, GTC by GAA, and GTG by GCG, respectively. Mutants of Glu45 are: E45A (GAG to GCG), E45D (GAG to GAT), E45K (GAG to AAG), E45Q (GAG to CAG). PCR amplification used 2.5 U *Pfu* DNA polymerase for 16 cycles (95°C, 45 s; 55°C, 1 min; 68°C, 15 min). A single DNA band of 4.9 kb as PCR product was observed on agarose gel. After cleavage of parent DNA by *Dpn*I digestion, mutated DNA was cloned into XL1 Blue cells. Mutated sequences were confirmed by DNA sequencing.

Expression and purification of mutants

Mutant proteins were purified as previously described [18,41]. Briefly, transformed cells were grown in LB medium containing 30 mg/l of ampicillin overnight. Harvested cells were lysed by lysozyme or using a French press, followed by solubilization of membrane proteins and heat treatment at 60°C to denature and remove most *E. coli* proteins. The heat denaturation step was omitted for temperature studies of cyclase activity and protein stability. Mutants were purified to homogeneity by eluting from Ni-NTA agarose (Qiagen) with 125 mM imidazole in 50 mM Na-phosphate, 300 mM NaCl and 10% glycerol at pH 7.0.

Enzyme labeling and activity assay

Purified mutants were labeled by [^{3}H]Ro48-8071 as previously described [17]. For enzyme assay, 50 μl purified SHC mutant was incubated with [^{14}C]squalene at 60°C for 45 min or 1 h. The reaction mixture was extracted with CH_2Cl_2 and analyzed by TLC. GC was carried out using an HP-5 capillary column (30 m, 0.32 mm i.d.) programmed at 250°C, 1 min; 5°C/min to 280°C; held at 280°C for 30 min.

Kinetic study

To measure kinetic parameters, 2, 4, 6, 8, or 10 μM [^{14}C]squalene (final conc.) was added and incubated with appropriate amounts of enzymes at 60°C for 1 h. The conversions of squalene to products were assayed by TLC and plotted according to the method of Lineweaver and Burk [42].

Substrate and product purification of the triple mutant

Oxidosqualene was prepared by treatment of squalene with *N*-bromosuccinimide in tetrahydrofuran/H_2O followed by reaction of bromohydrin with K_2CO_3 in methanol [43]. *E. coli* cell lysate for the triple mutant was incubated with (3*RS*)-OS at 50°C for 16 h. CH_2Cl_2 extract was purified on silica gel with EtOAc:hexane (1:1) to remove Triton X-100. Oxidosqualene and products were separated on silica gel, and products were then purified by preparative TLC.

Spectroscopic data for achilleol A and 3α-achilleol A

For achilleol A, which has the 3β-configuration, the ^{1}H NMR spectrum showed δ 4.72 (d, 1H, H-27a), 4.52 (d, 1H, H-27b), 3.59 (dd, J = 10.5, 4.5 Hz, 1H, H-3), 2.11 (m, 1H, H-1a), 1.82 (m, 1H, H-1b), 1.73 (m, 1H, H-2a), 1.43 (m, 1H, H-2b), 0.96 (s, 3H, H-26), 0.65 (s, 3H, H-25). For 3α-achilleol A, signals were obtained at δ 4.80 (d, 1H, H-27a), 4.54 (d, 1H, H-27b), 3.34 (m, 1H, H-3), 2.26 (m, 1H, H-1a), 1.93 (m, 1H, H-1b), 1.79 (m, 1H, H-2a), 1.58 (dd, 1H, H-5), 1.42 (m, 1H, H-2b), 0.91 (s, 3H, H-26), 0.82 (s, 3H, H-25). LRMS showed characteristic fragment ion at m/z 121 $[C_9H_{13}]^+$.

Supporting information available

Experimental details including ^{1}H NMR and LRMS of **6b** (hop-22(29)-en-3β-ol) and **6a** (hop-22(29)-en-3α-ol); ^{1}H NMR, ^{1}H–^{1}H COSY, HMQC and LRMS of the mixture of **7b** and **7a** (achilleol A and 3α-achilleol A).

Acknowledgements

The authors are indebted to Mr. J. Olsen (The University of Utah) for NMR measurement, and Dr. E.M. Rachlin (University of Utah) for MS measurement. We thank Dr. I. Abe (University of Shizuoka, Japan) for helpful discussions, Ms. M. Guardiola (University of Barcelona, Spain) for assistance with purification of several mutants, and Professor K. Poralla (Universität Tübingen, Germany) for providing the original *A. acidocaldarius* SHC clone. We also thank Drs. Abe and Y.F. Zheng (University of Utah) for synthesis of [^{3}H]Ro48-8071. This work was supported by the National Institutes of Health (Grant GM 44836 to G.D.P.).

References

1. Abe, I., Rohmer, M. & Prestwich, G.D. (1993). Enzymatic cyclization

of squalene and oxidosqualene to sterols and triterpenes. *Chem. Rev.* **93**, 2189–2206.
2. Abe, I. & Prestwich, G.D. (1998). Squalene epoxidase and oxidosqualene:lanosterol cyclase. Key enzymes in cholesterol biosynthesis. In *Comprehensive Natural Products Chemistry*. (Barton, D.H.R. & Nakanishi, K., eds), pp. 267-298, Elsevier, London.
3. Woodward, R.B. & Bloch, K. (1953). The cyclization of squalene in cholesterol synthesis. *J. Am. Chem. Soc.* **75**, 2023–2024.
4. Stork, G. & Burgstahler, A.W. (1955). The stereochemistry of polyene cyclization. *J. Am. Chem. Soc.* **77**, 5068–5077.
5. van Tamelen, E.E. (1982). Bioorganic characterization and mechanism of the 2,3-oxidosqualene–lanosterol conversion. *J. Am. Chem. Soc.* **104**, 6480–6481.
6. Abe, I. & Prestwich, G.D. (1998). Oxidosqualene cyclase inhibitors. *Drug Discov. Today* **3**, 389–390.
7. Lesburg, C.A., Caruthers, J.M., Paschall, C.M. & Christianson, D.W. (1998). Managing and manipulating carbocations in biology: terpenoid cyclase structure and mechanism. *Curr. Opin. Struct. Biol.* **8**, 695–703.
8. Wendt, K.U. & Schulz, G.E. (1998). Isoprenoid biosynthesis: manifold chemistry catalyzed by similar enzymes. *Structure* **6**, 127–133.
9. Merkofer, T., Pale-Grosdemange, C., Wendt, K.U., Rohmer, M. & Poralla, K. (1999). Altered product pattern of a squalene–hopene cyclase by mutagenesis of active site residues. *Tetrahedron Lett.* **40**, 2121–2124.
10. Pale-Grosdemange, C., Merkofer, T., Rohmer, M. & Poralla, K. (1999). Production of bicyclic and tricyclic triterpenes by mutated squalene–hopene cyclase. *Tetrahedron Lett.* **40**, 6009–6012.
11. Dougherty, D.A. (1996). Cation-π interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. *Science* **271**, 163–168.
12. Wendt, K.U., Poralla, K. & Schulz, G.E. (1997). Structure and function of a squalene cyclase. *Science* **277**, 1811–1815.
13. Wendt, K.U., Lenhart, A. & Schulz, G.E. (1999). The structure of the membrane protein squalene–hopene cyclase at 2.0 Å resolution. *J. Mol. Biol.* **286**, 175–187.
14. Feil, C., Sussmuth, R., Jung, G. & Poralla, K. (1996). Site-directed mutagenesis of putative active-site residues in squalene–hopene cyclase. *Eur. J. Biochem.* **242**, 51–55.
15. Perzl, M., Muller, P., Poralla, K. & Kannenberg, E.L. (1997). Squalene–hopene cyclase from *Bradyrhizobium japonicum*: Cloning, expression, sequence analysis and comparison to other triterpenoid cyclases. *Microbiology* **143**, 1235–1242.
16. Morand, O.H., Aebi, J.D., Dehmlow, H., Ji, Y.H., Gains, N., Lengsfeld, H. & Himber, J. (1997). Ro 48-8071, a new 2,3-oxidosqualene:lanosterol cyclase inhibitor lowering plasma cholesterol in hamsters, squirrel monkeys, and minipigs: Comparison to simvastatin. *J. Lipid Res.* **38**, 373–390.
17. Abe, I., Zheng, Y.F. & Prestwich, G.D. (1998). Photoaffinity labeling of oxidosqualene cyclase and squalene cyclase by a benzophenone-containing inhibitor. *Biochemistry* **37**, 5779–5784.
18. Dang, T., Abe, I., Zheng, Y.F. & Prestwich, G.D. (1999). The binding site for an inhibitor of squalene:hopene cyclase determined using photoaffinity labeling and molecular modeling. *Chem. Biol.* **6**, 333–341.
19. Abe, I. & Prestwich, G.D. (1994). Active site mapping of affinity-labeled rat oxidosqualene cyclase. *J. Biol. Chem.* **269**, 802–804.
20. Corey, E.J., Cheng, H.M., Baker, C.H., Matsuda, S.P.T., Li, D. & Song, X.L. (1997). Studies on the substrate binding segments and catalytic action of lanosterol synthase. Affinity labeling with carbocations derived from mechanism-based analogs of 2,3-oxidosqualene and site-directed mutagenesis probes. *J. Am. Chem. Soc.* **119**, 1289–1296.
21. Rohmer, M., Anding, C. & Ourisson, G. (1980). Non-specific biosynthesis of hopane triterpenes by a cell-free system from *Acetobacter pasteurianum*. *Eur. J. Biochem.* **112**, 541–547.
22. Abe, I. & Rohmer, M. (1994). Enzymic cyclization of 2,3-dihydrosqualene and squalene 2,3-epoxide by squalene cyclases – from pentacyclic to tetracyclic triterpenes. *J. Chem. Soc. Perkin Trans.* **1**, 783–791.
23. Xiao, X.-y. (1991). Ph.D. Dissertation, State University of New York, Stony Brook, NY.
24. Abe, I., Dang, T., Zheng, Y.-F., Madden, B.A., Feil, C., Poralla, K. & Prestwich, G.D. (1997). Cyclization of (3*S*)29-methylidene-2,3-oxidosqualene by bacterial squalene:hopene cyclase: irreversible enzyme inactivation and isolation of an unnatural dammarenoid. *J. Am. Chem. Soc.* **119**, 11333–11334.
25. Abdel-Mogib, M. (1999). A lupane triterpenoid from *Maerua oblongifolia*. *Phytochemistry* **51**, 445–448.
26. Barrero, A.F., Alvarez-Manzaneda Roldan, E.J. & Alvarez-Manzaneda, R. (1989). Achilleol A: a new monocyclic triterpene skeleton from *Achillea odorata* L. *Tetrahedron Lett.* **30**, 3351–3352.
27. Akihisa, T., Arai, K., Kimura, Y., Koike, K., Kokke, W.C.M.C., Shibata, T. & Nikaido, T. (1999). Camelliols A–C, three novel incompletely cyclized triterpene alcohols from sasanqua oil (*Camellia sasanqua*). *J. Nat. Prod.* **62**, 265–268.
28. Ochs, D., Tappe, C.H., Gärtner, P., Kellner, R. & Poralla, K. (1990). Properties of purified squalene-hopene cyclase from *Bacillus acidocaldarius*. *Eur. J. Biochem.* **194**, 75–80.
29. Corey, E.J., Cheng, H., Baker, C.H., Matsuda, S.P.T., Li, D. & Song, X. (1997). Methodology for the preparation of pure recombinant *S. cerevisiae* lanosterol synthase using a baculovirus expression system. Evidence that oxirane cleavage and A-ring formation are concerted in the biosynthesis of lanosterol from 2,3-oxidosqualene. *J. Am. Chem. Soc.* **119**, 1277–1288.
30. Jenson, C. & Jorgensen, W.L. (1997). Computational investigations of carbenium ion reactions relevant to sterol biosynthesis. *J. Am. Chem. Soc.* **119**, 10846–10854.
31. Gao, D.Q., Pan, Y.K., Byun, K. & Gao, J.L. (1998). Theoretical evidence for a concerted mechanism of the oxirane cleavage and A-ring formation in oxidosqualene cyclization. *J. Am. Chem. Soc.* **120**, 4045–4046.
32. Hart, E.A., Hua, L., Darr, L.B., Wilson, W.K., Pang, J.H. & Matsuda, S.P.T. (1999). Directed evolution to investigate steric control of enzymatic oxidosqualene cyclization. An isoleucine-to-valine mutation in cycloartenol synthase allows lanosterol and parkeol biosynthesis. *J. Am. Chem. Soc.* **121**, 9887–9888.
33. Joubert, B.M., Hua, L. & Matsuda, S.P.T. (2000). Steric bulk at position 454 in *Saccharomyces cerevisiae* lanosterol synthase influences B-ring formation but not deprotonation. *Org. Lett.* **2**, 339–341.
34. Kushiro, T., Shibuya, M. & Ebizuka, Y. (1999). Chimeric triterpene synthase. A possible model for multifunctional triterpene synthase. *J. Am. Chem. Soc.* **121**, 1208–1216.
35. Robustell, B., Abe, I. & Prestwich, G.D. (1998). Synthesis and enzymatic cyclization of (3*S*)11-fluoro-2,3-oxidosqualene. *Tetrahedron Lett.* **39**, 957–960.
36. Robustell, B.J., Abe, I. & Prestwich, G.D. (1998). Synthesis and enzymatic cyclization of (3*S*)-14-fluoro-2,3-oxidosqualene. *Tetrahedron Lett.* **39**, 9385–9388.
37. Füll, C. & Poralla, K. (2000). Conserved tyr residues determine functions of *Alicyclobacillus acidocaldarius* squalene–hopene cyclase. *FEMS Microbiol. Lett.* **183**, 221–224.
38. Sato, T. & Hoshino, T. (1999). Functional analysis of the DXDDTA motif in squalene–hopene cyclase by site-directed mutagenesis experiments: initiation site of the polycyclization reaction and stabilization site of the carbocation intermediate of the initially cyclized A-ring. *Biosci. Biotechnol. Biochem.* **63**, 2189–2198.
39. Bouvier, F., d'Harlingue, A. & Camara, B. (1997). Molecular analysis of carotenoid cyclase inhibition. *Arch. Biochem. Biophys.* **346**, 53–64.
40. Masson, P., Fortier, P.L., Albaret, C., Froment, M.T., Bartels, C.F. & Lockridge, O. (1997). Aging of di-isopropyl-phosphorylated human butyrylcholinesterase. *Biochem. J.* **327**, 601–607.
41. Ochs, D., Kaletta, C., Entian, K.-D., Beck-Sickinger, A. & Poralla, K. (1992). Cloning, expression, and sequencing of squalene–hopene cyclase, a key enzyme in triterpenoid metabolism. *J. Bacteriol.* **174**, 298–302.
42. Lineweaver, H. & Burk, D. (1934). The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**, 658–666.
43. Nadeau, R.G. & Hanzlik, R.P. (1969). Synthesis of labeled squalene and squalene 2,3-oxide. *Methods Enzymol.* **15**, 346–351.
44. Sato, T. & Hoshino, T. (1999). Kinetic studies on the function of all the conserved tryptophans involved inside and outside the QW motifs of squalene–hopene cyclase: Stabilizing effect of the protein structure against thermal denaturation. *Biosci. Biotechnol. Biochem.* **63**, 1171–1180.